United States Environmental Protection Agency

LABORATORY ANIMAL PROJECT REVIEW

Please note:

1. All information in this LAPR is considered privileged and confidential by the IACUC and regulatory authorities.
2. Approved LAPRs are subject to release to the public under the Freedom of Information Act (FOIA). Do not include proprietary or classified information in the LAPR.
3. An approved LAPR is valid for three years.

LAPR Information

LAPR Title:	Adrenal-derived stress hormones and air pollutant-induced lung injury and inflammation in rats
LAPR Number:	19-05-002
Principal Investigator	Exemption 6
Author of this Document:	Exemption 6 /RTP/USEPA/US
Date Originated:	05/09/2016
LAPR Expiration Date:	05/31/2019
Agenda Date:	05/18/2016
Date Approved:	05/27/2016
Date Closed:	

APPROVALS

APPROVER	NAME	APPROVAL DATE	COMMENTS	
	Exemption 6 Exemption 6 Exemption 6 Exemption 6 TP/USEPA/US by Exemption 6 /RTP/USEPA/US	05/27/2016	DMR	
	Exemption 6 Exemption 6 Exemption 6 RTP/USEPA/US by Exemption 6 /RTP/USEPA/US	05/27/2016	DMR	

Administrative Information

1. Project Title (no abbreviations, include species):
Adrenal-derived stress hormones and air pollutant-induced lung injury and inflammation in rats

Is this a continuing study with a previously approved LAPR?
Yes

Please provide the previous LAPR# 16-03-003

2. Programatic Information

a. What Program does this LAPR support? Please provide the Research Program, Project, Task Number and Title.
ACE PEP-1 and PEP-2

b. What is the Quality Assurance Project Plan (QAPP) covering this project?
QAPP-NHEERL-RTP/EPHD/CIB[redacted]/2016-001-r0

3. EPA Principal Investigator/Responsible Employee:

Principal Investigator	***Phone Number***	***Division***	***Mail Drop***
[redacted]	[redacted]	EPHD	MD
	Lotus Notes Address	***Branch***	
	[redacted]/RTP/USEPA/US	CIB	

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Alternate Contact	***Phone Number***	***Division***	***Mail Drop***
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	[redacted]/RTP/USEPA/US	CIB	

SECTION A - Description of Project

1. Explain the study objective(s) in <u>non-technical language</u> such that it is understandable by non-scientific persons. <u>Explain how the benefits from the knowledge gained from this research outweigh the costs to the animals used in this research.</u> If this is a continuing study from a previous LAPR, briefly justify the continuation. Please spell out all acronyms and abbreviations with their initial use.

A flight-or-fight stress response is induced in the body when a physical or psychological stress situation is encountered, for example fear, injury, burn or infection. This hormonal stress response is mediated by the central nervous system (CNS) and involves stimulation of sympathetic nervous system and hypothalamus-pituitary-adrenal (HPA)-axis, and the subsequent release of stress hormones from adrenal glands namely epinephrine, norepinephrine (also known as adrenaline and noradrenaline) and corticosterone (a steroid hormone). These stress hormones through their receptors (adrenergic and glucocorticoid) affect two survival mechanisms – metabolism and immune response in a dynamic manner in many organs in the body, such that the energy source and immune cells are mobilized from their depots and directed to the affected tissues to combat injury or danger signals and reestablish normal homeostasis. Virtually all organs are involved in orchestrating this homeostatic response to stress.

Even though we have known that pollutants that are inhaled by the lung are linked to effects in many organ systems and many types of chronic diseases such as cardiovascular, neurological and diabetes, the involvement of this hormonal stress response has not been implicated in the field of air pollution toxicology. Through the recent studies conducted under our previous LAPR (16-03-003), we have noted that inhalation of ozone induces this classical stress response and affects metabolism in many organs in the body in rats and humans Exemption 6 Exemption 6 et al., 2015; 2016a). We also showed that if the source of these stress hormones is removed through adrenalectomy surgeries, we eliminated not only metabolic effects of ozone but also pulmonary effects Exemption 6 Exemption 6 et al., 2016b), suggesting that adrenal-derived stress hormones are needed for metabolic and immune responses mediated by ozone. Our studies question the accepted paradigm that ozone being an oxidant causes lung injury by its interaction with lung lining components locally and stimulates inflammatory cell recruitment to the lung. The role of these stress hormones in mediating air pollution health effects becomes important, because in the field of medicine the stress response mechanisms are manipulated through the use of adrenergic and glucocorticoid receptor antagonists and agonists to counter pathological conditions. For example, steroid hormones are injected to reduce chronic inflammation and beta adrenergic blockers are employed in many pulmonary inflammatory conditions. Thus, it is important to understand how these stress hormones mediate organ-specific immune and metabolic effects of air pollutants to develop effective mitigation strategies and understand potential contraindications for those under the treatment of steroid hormones and beta blockers.

In this project we like to address how ozone-induced lung injury and inflammation are controlled by the release of stress hormones from adrenal glands using adrenalectomy surgeries and treating animals with steroid and adrenergic receptor agonists. This understanding is important in determining what interventional and mitigation strategies are needed to reduce stress related complications.

2. Scientific rationale for proposed animal use.

a. Why is the use of animals necessary?

The use of animals is necessary in order to understand the complex systemic changes in multiple organs that occur after exposure to pollutants in animal models. We have demonstrated that the exposure of healthy rats to pollutants can produce profound systemic response through CNS stress pathway. In vitro experimental approach will not be appropriate for determination of systemic health effects and assessing neuronal stress effects on multiple organs. Following a bibliographic search in Pubmed, no validated accepted non-animal methods have been identified to properly mimic inhalation exposures and the subsequent assessment of complex systemic metabolic response. Further, the use of an animal model allows for the undertaking of mechanistic studies including the change to the multiple organs which may provide important information on the disease risk.

b. Justify the species requested:

National Institute of Health guidelines recommends the use of rats to study human cardiovascular and metabolic diseases. Rat has been also a preferred animal model for the study of cardiovascular injury from air pollution. Moreover systemic disorders are better modeled in rats than in mice or in lower vertebrates. Due to the longstanding use of rats for toxicological, cardiovascular, and metabolic studies the necessary databases, reagents, and species-specific assays have been developed, verified to be accurate, and are commercially available. Toxicology data are available for rats to correlate findings of air pollution health effects. We have done a number of toxicological studies using Wistar Kyoto (WKY) rats. Since the studies anticipated under this LAPR involve continuation of our

previous studies using WKY rats for examining cardiovascular and metabolic health effects, we propose using this strain of rats to understand how stress response mediates systemic and pulmonary effects of ozone.

3. How was it determined that this study is not unnecessary duplication?

Pubmed and literature searches in Google Scholar performed in May 2016 failed to identify any published studies that investigated neurohormonal stress response in ozone induced systemic metabolic and pulmonary injury inflammation alterations. Using search terms, "ozone" and "stress" and "metabolism" and "beta receptors" yielded three references, one involving brain lipid peroxidation and estradiol in female rats, one involving guinea pig trachea and one involving a study in fish. However, using search terms, "ozone" and "stress" and "metabolism" and "glucocorticoid receptors" yielded no references. The use of ozone and adrenalectomy yielded two references, one is our own where we showed reversal of metabolic effects by adrenalectomy and the other one where thymus atrophy was studied. We have the current knowledge through direct interaction with scientists in the field and through scientific meetings, and believe that our experiments are not duplicated anywhere else. Our literature review through Pubmed and criteria document of air pollution studies revealed no publication involving immune response with ozone using adrenalectomized rats.

SECTION B - In Vivo Procedures

1. Briefly describe the experimental design. Include descriptions of the age, weight and sex of the animals. Supplementary information may be attached at the end of the LAPR, but please include critical information within the body of the LAPR.

This project will involve adrenalectomy, subsequent treatment of rats with stress hormone agonists and ozone exposure.

The study will examine how inhaled ozone can induce a CNS-mediated hormonal stress response and downstream systemic metabolic and immune effects using adrenalectomy and pharmacological interventions of stress hormone replenishment in rats. We will use 12-16 weeks old male Wistar Kyoto rats (300-350 grams) for this protocol. Our prior study under LAPR 16-03-003 showed that adrenalectomy resulted in diminution of two circulating stress hormones, epinephrine and corticosterone, and also ozone-induced systemic and pulmonary effects. In this study we will examine how ozone immune effects are modulated by adrenalectomy. In addition, we will assess if replenishing stress hormones by beta adrenergic plus glucocorticoid receptor agonists (epinephrine plus corticosterone mimics), Clenbuterol plus dexamethasone, respectively in adrenalectomized rats will result in reappearance of ozone systemic and pulmonary effects. Since adrenalectomy can result in many different physiological effects besides reducing circulating stress hormones, epinephrine and corticosterone, it is important to confirm the role of circulating stress hormones in mediating systemic and pulmonary effects of ozone. Therefore, the study design will include SHAM or total bilateral adrenalectomy surgeries followed by treatment of rats with vehicles or Clenbuterol plus dexamethasone for 2 or 3 consecutive days, and then air or 0.8 ppm ozone exposure (4hours/day) for 1 or 2 consecutive days. The control SHAM surgery group includes rats, which undergo similar surgical procedures as adrenalectomy except that adrenals are not removed. Administration of Clenbuterol and dexamethasone are expected to mimic the effect of circulating epinephrine and corticosterone, respectively on lungs of adrenalectomized rats. In SHAM rats these drugs might diminish ozone induced increases in circulating stress hormones. We have noted that ozone exposure abolishes insulin release into the circulation in response to glucose injection. It has been shown that epinephrine plays a role in blunting the insulin secretion. Since we are examining the role of beta receptor agonist, we believe that it is important to determine if this metabolic effect of ozone is impacted by beta-2 adrenergic agonist and adrenalectomy.

Experimental protocol:
Adrenalectomy study will include following 2 critical aspects (please see attached experimental schema for each week's protocol):

1) Surgery, treatments and exposure timing:

Day 1: SHAM or adrenalectomy surgery (3-5 days recovery).
Day 4-8: Vehicles or Clenbuterol plus dexamethasone treatments begin for two or three consecutive days.

Day 5-8: Air or 0.8 ppm ozone exposure (4hours/day) for either 1 day or two consecutive days. 1-day exposure time point to obtain samples without manipulations of GTT or tail bleed, and maximum gene expression, and 2-day exposure to allow for all these measurements on day 1 while capturing peak of ozone effects on the lung on day 2.

2) Experimental groups.

Surgeries x 2: SHAM and adrenalectomy

Drug treatments x 2: Vehicles or Clenbuterol plus dexamethasone. Rats will be treated with vehicle for Clenbuterol (saline, 1 mL/kg body weight, subcutaneous) plus vehicle for dexamethasone (1% carboxymethylcellulose in saline, 1 mL/kg intraperitoneal) or Clenbuterol (1.0 mg/kg body weight/mL saline, subcutaneous) plus dexamethasone (5 mg/kg/mL 1% carboxymethyl cellulose in saline, intraperitoneal) once a day (in the morning between 6 am-7 am) for 2-3 consecutive days. The drug treatment will begin one day before air or ozone exposure and will continue each day of exposure for a total of two treatments for 1-day animals and 3 treatments for 2-day animals.

In life measurements in 2-day exposure animals (none in 1-day animals):

1) Body temperature and whole body plethysmography: One day prior to and immediately after both days of air or ozone exposure (within 30 minutes) body temperature measurement will be made (intrascapular, tail base, and hind leg) using an infrared temperature scanner since temperature deregulation is expected after adrenalectomy. During this time animals will also undergo whole body plethysmography measurements using EMKA –Buxco system to determine effects of ozone on breathing parameters in SHAM and adrenalectomized rats.

2) GTT and tail blood collection for insulin: Immediately after (within 30 minutes) of first day of air or ozone exposure the 2-day animals will undergo GTT and tail blood (~200 microliters) for insulin measurement. Blood glucose for GTT will be measured at baseline and every 30 min thereafter until 120 minutes, whereas, tail blood will be collected only at baseline and 30 min post glucose injection.

Necropsy protocol for 1-day and 2-day animals: After euthanasia, terminal blood collection will be done for measurement of stress hormones, circulating metabolites and inflammatory markers as well as immune cells. Several tissues will be collected including bone marrow, spleen, thymus, adipose tissues, liver, muscle and brain, and processed for metabolic changes and changes in immune cell trafficking using various staining techniques. In addition, the lungs will be lavaged and processed for isolation of immune cells and histopathology for determining inflammation. This information will be critical in examining the role of stress hormones in meditating immune and metabolic effects in various organs.

2. Justify the number of animals. Include explanation (e.g., biological, statistical, regulatory rationale) for the number of animals needed for each treatment group, and the overall number requested for the duration of the LAPR.

A total of 192 male Wistar Kyoto (WKY) rats are requested for the first experiment involving adrenalectomy. For appropriate statistical power each group of animals will include a minimum of 8 rats. Since Charles River Surgeons request additional ~30% animals to be available to account for any loss of animals during surgery or post surgery complications we will request n=12 rats per group to be ordered for this experiment. Considering the following protocol 192 rats will be required for adrenalectomy studies: The animals, if left over that will not undergo surgeries, will be used as cage controls.

a) Surgeries x 2: SHAM and adrenalectomy
b) Drug treatments x 2: Vehicles and Clenbuterol plus dexamethasone
c) Exposures x 2: Air and 0.8 ppm ozone
d) Time points x 2: 1-day and 2-day
e) Total number of animals per group=12.

Thus, a total of 192 WKY rats will be needed for this protocol. The use of same animals for multiple measures and monitoring assessments during this study reduces the need to include multiple sets of animals. Further, the robust amount of tissues that will be obtained and preserved allow for a variety of ex vivo analyses. Such samples can later be compared with research that involves pharmacological interventions, ozone and adult disease risk and ultimately reduces the need to repeat such experiments in the future. Of 192 rats, 96, which will undergo surgery and then subjected to air exposure will be considered under Category D (for surgery) and 96 rats, which will undergo surgery and then subjected to ozone exposure will be considered under Category E (for surgery plus ozone).

3. State how many animals over the study period are expected to be used under the following three categories of pain/distress (USDA nomenclature as defined in the instructions): Please enter numbers only.

Categories	Adults	Offspring
C) Minimal, transient, or no pain/distress:		
D) Potential pain/distress relieved by appropriate measures:	96	
E) Unrelieved pain/distress:	96	

4. Does this LAPR include any of the following:

☐ Restraint (>15 Minutes) ☒ Survival surgery

☒ Food and/or water restriction (>6 Hours) ☐ Non-survival surgery

a. Please provide a scientific justification. Describe how animals will be monitored, how health status will be tracked, and what records will be maintained.

The surgical intervention of adrenalectomy will allow for complete elimination of circulating stress hormones and examination of its influence on ozone effects. No pharmaceutical approaches will allow for nearly complete elimination of these stress hormones. This is very critical in understanding how these hormones play a crucial role in mediating ozone-induced metabolic and pulmonary effects. No other approaches will allow to test the hypothesis that these hormones are essential in mediating air pollution effects.

After surgery rats will be continuously monitored until fully awake and placed on a heating pad to maintain body temperature. The adrenalectomized rats will be maintained on a bottled water containing 0.9% sodium chloride post-surgery. We will use edible sodium chloride to prepare drinking water. Powdered food will be provided in feeding cups post-surgery for all animals till necropsy except for the fasting periods. Although, no deaths are anticipated due to adrenalectomy or SHAM procedures, rats will be closely monitored during the postoperative period. After the surgery, animals will have rest for 2-4 full days before the next procedure. No other special husbandry requirements are needed for adrenalectomized rats. SHAM operated rats will be treated in identical manner as adrenalectomized animals but will be provided regular tap water in water bottles. We will closely monitor these rats and will provide additional analgesic treatment as recommended by attending veterinarian.

Exemption 6Exemption 6Exemption 6Exemption 6 will be monitoring animals twice daily after their surgery (during recovery and experimentation periods including weekends). It is not expected that for adrenalectomy protocol, animals will suffer dehydration or weight loss issues based on our earlier study. Since these rats are going to be used within 1 week, this too will minimize adverse health consequences upon extended period. Nevertheless, the attending veterinarian will be notified immediately and if advised, subcutaneous saline bolus will be administered as suggested (10 ml/kg).

Attending veterinarian will be consulted and recommendations will be followed for animals displaying unexpected weight loss of 20% or greater. All animals will be housed 2/cage with beta chips bedding and nesting material EnviroDry in A building 5th floor during non-exposure periods. After adrenalectomy and SHAM surgeries, performed under LAPR 16-03-003, there were no incidences of dehiscence as the incision was on the dorsal surface of animals. Nevertheless, attending veterinarian will be notified if any incidence of dehiscence is observed, and the recommendations will be followed for the follow-up procedures and treatments.

Food restriction for >6 hours: The performance of glucose tolerance test and the assessment of metabolic markers requires prior 6-8 hours fasting in rodent studies to obtain stabilized baseline values for metabolites which are highly influenced by food intake. Glucose tolerance testing will be done immediately after first day of air or ozone exposure in 2-day exposure group for which fasting glucose measurements are needed. Animals will be

fasted during 4 hour ozone exposure and then during ~2.5 hours glucose tolerance testing. Animals will be monitored hourly during ozone exposure for signs of discomfort and then continuously through glucose tolerance testing during which a large bolus of glucose is injected, which might eliminate some effect of food deprivation.

5. Category C procedures. Describe each procedure separately, include details on the following:

a. Treatments (e.g., dosages, duration of exposure, route, volume, frequency):

1) Saline: Pharmaceutical grade sterile saline will be injected subcutaneously (1 mL/kg) in vehicle control rats one day prior to the start of exposure, the first day prior to exposure and second day prior to exposure.

2) Clenbuterol: This beta adrenergic receptor agonist, will mimic the effect of circulating epinephrine, especially in the lung. Clenbuterol will be dissolved in saline at 1.0 mg/mL and administered subcutaneously at 1mL/kg dose, one day prior to the start of exposure, the first day prior to exposure and second day prior to exposure. Clenbuterol is widely employed in experimental studies in animals including rats. Many studies have involved the use of this or higher concentrations of the drug and several days of treatment regimen. Our LAPR 19-02-002 involves its use to determine the influence on the lung of normal rats exposed to ozone.

3) 1% carboxymethylcellulose in saline: 1% carboxymethylcellulose in pharmaceutical grade sterile saline will be injected intraperitoneally (1 mL/kg) as vehicle control for dexamethasone in rats, one day prior to the start of exposure, the first day prior to exposure and second day prior to exposure.

4) Dexamethasone, 5 mg/kg in 1 mL 1% carboxymethylcellulose in saline will be injected intraperitoneally (1 mL/kg) rats, one day prior to the start of exposure, the first day prior to exposure and second day prior to exposure.
Dexamethasone is widely used in research and it will mimic the action of corticosterone in rats.

5) Pharmaceutical grade glucose for glucose tolerance testing (GTT): SHAM and adrenalectomized rats assigned 2-day ozone exposure will undergo GTT after their first day of ozone exposure. During GTT, after the baseline blood glucose measurement, pharmaceutical grade glucose will be injected intraperitoneally (maximum of 2 grams/kg body weight/10mL pharmaceutical grade saline). The 10ml/kg body weight volume has been used in published studies for rats. We will purchase pharmaceutical grade sterile glucose solution (40% concentration) from Sigma Aldrich and dilute to 20% using pharmaceutical grade saline. Glucose solution will be made fresh each time using new pharmaceutical grade saline vials. Sterile syringe and needles will be used for each rat for intraperitoneal injection. In our prior studies animals showed no signs of peritonitis or infections in the abdomen after repeated GTT over a three month period. Their weight gains were not affected by GTT. The scientific staff involved in the study will watch for signs of abdominal pain such as lateral or vertical stretching, and weight loss daily until the necropsy.

No deleterious effects of these drug treatments are expected in animals. All drug treatments will occur for a maximum of 3 days, once per day. After the first treatment rats will be monitored for 2-3 hours for possible discomfort. Then during each subsequent treatment in the morning prior to exposure rats will be monitored for possible discomfort and signs of weight loss. Attending veterinarian will be notified for advice if significant weight loss is observed.

b. Survival Blood Collections (method, volume, frequency):

For all rats undergoing GTT test (2-day exposure groups), each will have 7 blood samples taken. The 2 tail pricks for large blood volume will be for insulin measurement and the 5 tail pricks, one microliter each, are for glucose measurement. Blood collection for glucose measurement during GTT: For animals assigned 2-day exposure, the baseline blood glucose levels will be measured following ~6 hours fasting. Blood will be collected by pricking the tip of the tail with a 25 gauge sterile needle following wiping with an alcohol swab and clean dry gauze. About 1 microliter blood droplet will be brought into contact with the glucometer strip (attached to Bayer Contour Glucometer) (0.6 micro liter of blood is aspirated in the strip). Glucose is measured within three seconds and recorded. Once baseline glucose is measured, pharmaceutical grade glucose will then be injected intraperitoneally (2 grams/kg body weight/10mL) and blood glucose will be measured at 30 min interval for four times in each rat. For GTT, each rat will have a total of 5 glucose measurements (baseline plus four times following intraperitoneal injections of glucose). During GTT tail vein blood collection will be done as indicated above.

Timeline for GTT: GTT is performed right after exposure (~11am start time) as stated below:

-Blood glucose measured by tail prick at 0 min and tail vein blood collected as below
-Glucose injected intraperitoneally right after the zero minute blood glucose testing
-Glucose measured by tail prick at 30 min and tail vein blood collected as below
-Glucose measured by tail prick at 60 min
-Glucose measured by tail prick at 90 min
-Glucose measured by tail prick at 120 min

Blood collection for measurement of insulin will be performed during GTT for only 2-day exposure group. As specified above, two blood draws will be done on each rat; one at baseline and one 30 minutes post glucose injection. Rats will be restrained for ~3-5 minutes in a conical nose-only restrainer that we use for inhalation studies. These tubes will be anchored by straps at the edge of lab platform using an anchoring device custom made for this purpose. The rat tails will be warmed and cleaned with warm wet cloth. The tail will be wiped cleaned and lateral vein will be visualized. Winged infusion needles (21-25G) will be used to insert in the tail vein. Blood will be allowed to drip in Microtainer tube with a serum separator plug (~200 microliter). The hemostasis will be achieved by pressure and clean sterile gauze. Thirty minutes after injection of pharmaceutical grade glucose for GTT (2g/kg/10mL; intraperitoneal) the second blood collection on same rat will be done as shown above. The hemostasis will be achieved as above by pressure and clean sterile gauze. **Exemption 6** and I have performed this technique, under LAPR 16-03-003, amendment #12. Since blood collection will occur two times on same animal, we will alternate between right and left tail vein (~3 inch from the tip of the tail).

c. Testing methods (including non-stressful dietary restrictions/modifications, mild non-damaging electric shock):
Respiratory monitoring using whole body Plethysmography - EMKA/Buxco system: Whole body plethysmography measurements will be performed three times on rats assigned to 2-day group. Whole body plethysmography will be performed one day prior to the start of exposure, the first day after exposure and second day after exposure. All plethysmography measurements will be done at ~11 am in the morning to reduce diurnal variation. Breathing parameters will be measured using EMKA software and Buxco unit. Breathing parameters are monitored in freely moving rats. No restrain is used. Rats are placed in plethysmography chambers while pressure parameters are collected to compute breathing frequency, minute volume, respiratory time and enhanced pause before and after exposures. The rats are placed in a whole-body plethysmography for 5-10 min. We have routinely used this duration for acquisition of breathing parameters which has been adequate for stabilization and recording. No restraint or other stresses are involved in this process. This measurement allows in depth evaluation of lung health in unrestrained freely moving rats.

Body temperature measurement: One day prior and immediately after both days of air or ozone exposure (within 30 minutes during plethysmography) body temperature will be measured (intrascapular, tail base, and hind leg) using an infrared temperature scanner immediately before and following exposures since temperature deregulation is expected after adrenalectomy. This is non invasive procedures and the scanner does not touch animals. During this time animals will also undergo whole body plethysmography measurements as shown above.

d. Animal restraint and confinement beyond routine housing and handling. Include a description of the type of restraint device, acclimation to device, duration of restraint:
Nose-only tube restraint for tail vein blood collection: Rats will be acclimatized to conical plastic nose-only inhalation tubes for 5 minutes each day (designed for nose-only inhalation purpose) for 2 days prior to collection of blood. Animals will be placed in the nose-only restraining tubes on the holding rack during acclimation period. Then rats will be restrained for a period of no more than 5 minutes to nose-only tubes during blood collection by tail vein.

e. Breeding for experimental purposes (e.g. length of pairing, number of generations):
None.

f. Describe how animals will be identified and monitored. Include description of identification procedures. (For example, if transponders are used, how are the animals prepared?) Include frequency of observations and by whom:

Each animal will be identified by a unique identification number marked using permanent ink pen on tail and all cages will be labelled with details of animal numbers, treatments and exposure conditions.

6. Non-surgical Category D or E procedures. Describe each procedure separately, include details on the following (Also fill in Section B.9).

a. Treatments (e.g. dosages, duration of exposure, route, volume, frequency):

Ozone exposure is classified as Category E procedure. Rats will be exposed to 0.8 ppm ozone, 4hrs per day, for either 1 or 2 consecutive days. Ozone at this concentration in Wistar Kyoto rat produces lung inflammation, hypothermia, and a stress response which resolves on its own after one day upon discontinuation of exposure.

During air or ozone inhalation exposures of maximum of 4hrs/day (whole-body), rats are placed in individual stainless steel wire mesh cages (length,27.3 cms; width, 14.6 cms, and hight 17.75 cms), and food and water are withheld while the rats are being exposed. Ozone exposures will be done using whole body exposure system in large Hazelton 2000 Inhalation chambers (2 cubic meters internal volume, CH Technologies, Westwood, NJ) where each rat is placed in wire-mesh cages. The rats are weighed daily following each exposure and examined for any visible clinical signs of discomfort or poor health. The rats are also checked after each exposure when they are returned to home cages. All findings are recorded. We have done a number of ozone inhalation studies in the previous LAPR (#16-03-003).

b. Blood Collection (Provide a description of the procedure including method, volume, and frequency if appropriate. Indicate if the procedure is survival or terminal. Include preparatory methods, descriptions of incisions, etc.):

As indicated in section 5.b., in ozone-exposed (Category E) animals, survival blood collection for measurement of insulin secretion will be performed immediately following day 1 of ozone exposure (coordinated with GTT) for only 2d-ay exposure group. Blood will be collected for insulin measurement at baseline and immediately following 30 minutes after bolus glucose injection. ~200 microliter (each time) blood samples will be collected through tail vein in a serum collection tube.

Two blood draws will occur for serum insulin measurement, one before glucose injecton and a second 30 minutes post glucose injection. Rats will be restrained for ~3-5 minutes in a conical nose-only restrainer that we use for inhalation studies. These tubes will be anchored by straps at the edge of lab platform using an anchoring device custom made for this purpose. The rat tails will be warmed and cleaned with warm wet cloth. The tail will be wiped cleaned and lateral vein will be visualized. Winged infusion needles (21-25G) will be used to insert in the tail vein. Blood will be allowed to drip in Microtainer tube with a serum separator plug (~200 microliter during each draw). The hemostasis will be achieved by pressure and clean sterile gauze. The animals will be removed from the tubes and returned to their cages. Thirty minutes after injection of pharmaceutical grade glucose for GTT (2g/kg/10mL; intraperitoneal) the second blood collection on same rat will be done as shown above. The hemostasis will be achieved as above by pressure and clean sterile gauze. Then rats will be relocated in their respective cages. **Exemption 6** have performed this technique, under LAPR 16-03-003, amendment #12. Since blood collection will occur two times on same animal, we will alternate between right and left tail vein (~3 inch from the tip of the tail).

For GTT ~1 microliter blood is suctioned into the glucometer after a tail prick using a 20 gage sterile needle. This is done at baseline (0 min), and after 30 min, 60 min, 90 min, and 120 min of glucose injection during testing.

c. Testing methods:

Whole body Plethysmography using EMKA/Buxco system: As indicated in section 5.c., respiratory monitoring using whole body plethysmography measurements will be performed three times on rats assigned to 2-day group. Whole body plethysmography will be performed one day prior to the start of exposure, the first day after exposure and second day after exposure. All plethysmography measurements will be done at ~11 am in the morning to reduce diurnal variation. Breathing parameters will be measured using EMKA software. Breathing parameters are monitored in freely moving rats. No restrain is used. Rats are placed in plethysmography chambers while pressure parameters are collected to compute breathing frequency, minute volume, respiratory time and enhanced pause before and after exposures. The rats are placed in a whole-body plethysmography for 5-10 min. We have routinely used this duration for acquisition

of breathing parameters which has been adequate for stabilization and recording. No restraint or other stresses are involved in this process. This measurement allows in depth evaluation of lung health in unrestrained freely moving rats.

Body temperature measurement: One day prior and immediately after both days of air or ozone exposure (within 30 minutes) body temperature measurement will be made (intrascapular, tail base, and hind leg) using an infrared temperature scanner immediately before and following exposures since temperature deregulation is expected after adrenalectomy. The temperature probe will not touch animals. During this time animals will also undergo whole body plethysmography measurements as shown above.

d. Restrictions placed on the animals' basic needs (e.g., food and/or water restriction, light cycles, temperature). Provide details regarding the length of restriction. Describe the method(s) for assessing the health and well-being of the animals during restriction. (Amount of food or fluid earned during testing and amount freely given must be recorded and assessed to assure proper nutrition.):

Food restriction for >6 hours during ozone exposure and GTT: The performance of glucose tolerance test and the assessment of metabolic markers requires prior 6-8 hours fasting in rodent studies to obtain stabilized baseline values for metabolites which are highly influenced by food intake. GTT will be done immediately after first day of air or ozone exposure in 2-day exposure group for which fasting glucose measurements are needed. Animals will be fasted during 4 hour ozone exposure and then during ~2.5 hours glucose tolerance testing. Animals will be monitored hourly during ozone exposure for signs of discomfort and then continuously through GTT during which a large bolus of glucose is injected, which might eliminate some effect of food deprivation.

e. Describe how animals will be monitored (e.g., frequency of observations, by whom):

The animals with SHAM surgery and adrenalectomy that have undergone ozone exposure will be continuously monitored. During exposure, Exemption 6Exemption 6Exemption 6 Exemption 6 will monitor animals, at least once per hour for entire exposure duration. During post exposure period of up to 20 hours, rats will be monitored by Exemption 6Exemption 6Exemption 6 in the evening and then in the morning for visible signs of discomfort and weight loss. All animals will be monitored for signs of illness (huddling, isolation with ruffled coat, shivering, development of hindered movement, etc) and if any adverse effect is observed, we will consult with the staff veterinarian and follow the recommended protocol. Visual inspection of labored breathing and isolation will be carefully monitored. No weekend exposures are scheduled, and the animals will be necropsied within 48 hours of the start of the first ozone exposure. No significant weight loss due to ozone is expected in any of the experimental conditions.

f. Analgesia (Category D Procedures) - list drugs, dosages, route of administration and frequency:

No analgesia will be used for the procedures described in this section, however, analgesics will be used for surgical procedures as described in section 7.

g. If treatment-related deaths are expected, this must be thoroughly justified. Death as an endpoint is highly discouraged:

No procedure-related deaths are expected for this category.

7. Surgical Category D and E procedures. Indicate if the surgery is survival or terminal. Describe each surgical procedure separately, include details on the following (Also fill in Section B.9)

a. Complete description of surgical procedure including presurgical preparation, aseptic technique, surgical closure, etc:

Rats will be purchased and housed in EPA animal facility until they reach 12-16 weeks age. The surgeries will be performed at 12-16 weeks of age. The surgeries will be performed on 3rd floor procedure rooms Exemption 6 Exemption 6. The room will be reserved in advance and the door signs will restrict entry of non-essential personnel to the suite during surgeries.

Adrenalectomy Surgery: Charles River surgeons will use their IACUC approved protocol to perform aseptic surgery. Surgeries will be performed on two Fridays; for the for the adrenalectomy study. These surgeons will have not been in contact with other rodents or other animal facility on the same day they enter the EPA animal facility. They will use their institutionally approved procedures and quality requirements for aseptic

procedures. The needs of the surgeon/s will be met and that they will be escorted to the procedure room.

We expect the surgeries to be performed following something along the following lines. The rats will be anesthetized using injectable anesthesia (ketamine/xylazine). Animals will be checked for anesthetic level by "lack of response to several firm toe pinches. When anesthetized rats will be injected with Buprenorphine analgesic subcutaneously at 0.02 mg/mL (diluted with saline). Eye ointment will be applied to prevent drying. Aseptic technique will be observed for the surgeries, including the use of sterilized instruments and suture material. Masks and gloves will be worn during the surgery. All surgical procedures will be performed by Charles River surgeons.

Animals will have dorsal back fur clipped, and will be scrubbed with surgical soap and alcohol in alternating scrubs 3 times. A sterile drape will be placed over the intended incision site. A dorsal approach will be made through the lumbar musculature and into the abdominal cavity. For adrenalectomies and sham operations, the surgeons will make dorsal skin incision, and locate each adrenal right above each kidney in the abdominal cavity. For total bilateral adrenalectomy, the whole adrenal gland from each side will be clipped off and surgically removed. For sham operation adrenals will not be removed. Upon rearrangement of organs within abdominal cavity, the incision will be closed using surgical staples. Sham surgeries are done in an identical manner except that the adrenals are not removed. Charles River will use 6-7 mm wound clips. These wound clips are generally removed after ~7-10 days of surgery but because these animals will be used within one week wound clips will not be removed. Animals will be allowed to recover over the heating pad while under continuous supervision. As recommended by Charles River surgeons and our veterinarian, we will use Meloxicam, 1.0 mg/kg, subcutaneous for analgesia when animals are awakening from anesthesia. Once fully awake and moving around, animals will be placed in their home cages with access to powdered food and 0.9% sodium chloride (saline) as drinking water for adrenalectomy rats and regular tap water for SHAM rats. Two additional subcutaneous injections of Buprenorphine will be given for analgesia, one in the evening of the day of surgery and one next day morning. The dose is 0.02 mg/kg given subcutaneously. Similar post surgery analgesia protocol will be employed for sham and adrenalectomized rats.

b. Anesthetic regimen (Drugs, dosages, volume, route of administration and delivery schedule). The use of paralytic or neuromuscular blocking agents w/o anesthesia is prohibited:

Rats will be anesthetized with intraperitoneal injection of 0.5-0.8 mL solution of ketamine plus xylazine (75-100 mg/kg ketamine plus 5-10 mg/kg xylazine, mixed fresh in the morning) and as needed. Additionally, during surgery isoflurane anesthesia will be used if any movement of limbs are noted during surgery. Induction cone will be ready to introduce additional anesthesia during surgery. The line fill be filled with 3% isoflurane (O2 flow at 0.8-1.0 L/min) and anesthesia will be maintained using 1%-3% isoflurane in oxygen delivered at 0.8-1.0 L/min delivered via nose cone if needed.

c. Postoperative care (thermal support, special feeding, responsible personnel, removal of sutures/staples, frequency and duration of monitoring including weekend and holiday care):

Immediately after surgery animals will be placed on the heating pads and continuously monitored for awakening from anesthesia. After compete awakening animals will be injected with Meloxicam (0.2 mg/kg, subcutaneously for analgesia and then transferred to their home cages. Two additional subcutaneous injections of Buprenorphine will be given for analgesia, one in the evening of the day of surgery and one next day morning. The dose is 0.02 mg/kg given subcutaneously. The adrenalectomized rats will be maintained on a bottled water containing 0.9% sodium chloride post-surgery. We will use edible sodium chloride to prepare drinking water. Powdered food will be provided in feeding cups post-surgery for all animals till necropsy except for the fasting periods. Although, no deaths are anticipated due to adrenalectomy or SHAM procedures, rats will be closely monitored during the postoperative period. After the surgery, animals will be allowed to recover from surgery for 3-5 days and then used for the study. No other special husbandry requirements are needed for adrenalectomized rats. Sham operated rats will be treated in identical manner as adrenalectomized animals but will be provided regular tap water in water bottles. We will closely monitor these rats and will provide additional analgesic treatment as recommended by attending veterinarian.

Exemption 6Exemption 6Exemption 6Exemption 6 will be monitoring animals twice daily after their surgery (during recovery and experimentation periods including weekends). It is not

expected that for adrenalectomy protocol, animals will suffer dehydration or weight loss issues based on our earlier study. Since these rats are going to be used within 1 week, this too will minimize adverse health consequences upon extended period. Nevertheless, the attending Veterinarian will be notified immediately and if advised by the attending veterinarian, subcutaneous saline bolus will be administered as suggested (10 ml/kg).

After adrenalectomy and SHAM surgeries, performed under LAPR 16-03-003, there were no incidences of dehiscence as the incision was on the dorsal surface of animals. Nevertheless, attending veterinarian will be notified if any incidences of dehiscence are observed, and the recommendations will be followed for the follow-up procedures and treatments. Dehiscence might require flushing of an open incision with sterile saline followed by a closure of wound using sterile surgical suture or application of surgical glue. This procedure will be performed in Exemption 6 procedure room using aseptic technique. Based on the recommendation of attending veterinarian, we will consider injectable analgesic and/or antibiotic treatment.

Attending veterinarian will be consulted and recommendations will be followed for animals displaying unexpected weight loss of 20% or greater. All animals will be housed 2/cage with beta chips bedding in A building during non-exposure periods.

d. Post operative analgesics (drugs, dosage, and volume and route of administration, frequency):
For both types of surgeries, upon awakening, rats will be injected subcutaneously with Meloxicam analgesic, 0.2 mg/kg dose in pharmaceutical grade saline. During the first 48 hours post-op, two additional doses of Buprenorphine (0.02 mg/mg, subcutaneously) be given, one in the evening of surgery day and one next day morning. Additional dose of Buprenorphine will be given if advised by attending veterinarian. If infection or swelling or redness is observed in rats, we will consult attending veterinarian **Exemption 6** to suggest appropriate protocol for injectable antibiotic treatment.

e. Will any animal be subject to more than one surgical procedure over the course of its lifetime, either here at NHEERL or elsewhere?
○ Yes ● No

f. Identify any surgical procedures performed at other institutions or by vendors:
SHAM and adrenalectomy surgeries will be performed by the CRL surgeons at the EPA facility, and details are provided in B.7.a.-d.

8. Humane interventions (for treatments/procedures in all categories).

a. What resultant effects, if any, do the investigators expect to see following procedures or treatment? Please include transitory as well as permanent effects. Examples might include lethargy, ataxia, salivation or tremors. Indicate the expected duration of these effects.
Unexpected weight loss of 20% or greater or visual signs of distress will result in immediate notification to the attending Veterinarian and consideration for euthanasia. No deleterious effects of adrenalectomy or SHAM surgeries are expected. When adrenalectomized animals are exposed to ozone, we have previously noted that ozone-induced lung injury, inflammation and systemic effects were diminished. Therefore, no ozone exposure-related complications are expected. Based on our previous adrenalectomy study, there were no incidences of seizures. Also, no significant hypoglycemia was noted in our animals during experimental period of 8 days as monitored during GTT (under LAPR 16-03-003, amendment #5; Exemption 6 et al., 2016). Nevertheless, in case of any of these complications are observed, we will consult with attending veterinarian and follow recommended protocol.

b. State the criteria for determining temporary or permanent removal of animals from the study. Describe actions to be taken in the event of deleterious effects from procedures or chemical exposures. Describe actions to be taken in the event of clinical health problems not caused by procedures or exposures.
If weight loss of >20% occurs overnight, animals will be isolated in a clean control atmosphere and observed for recovery trend, and may be transferred to the training colony if recovered. Any animals displaying signs of illness (huddling, isolation with ruffled exterior, shivering, development of hindered movement, etc) will be considered for permanent removal as per advice of the staff veterinarian. Visual inspection of labored breathing and isolation will be carefully monitored and if noted, the advice of the attending veterinarian will be followed for further action and possible euthanasia.

9. Alternatives to pain and distress (Category D and E Procedures only). Provide narrative regarding the

sources consulted to ascertain whether acceptable alternatives exist for potentially painful/distressful procedures. Include databases searched or other sources consulted, the date of the search and years covered by the search, and key words and/or search strategy used. Assistance with searches is available through the EPA Library Staff.

We have read many papers published in the past 20 years in the Pubmed (air pollution, adrenalectomy and rat) and through google search for alternatives to total and bilateral adrenalectomy to study pollution health effects. Chemical interventions have been often used for hormonal manipulations, but those interventions address specific hormone-mediated mechanisms and these intervention have non-specific effects. Most of the chemical interventions are focused for the therapeutic consideration and not in understanding the mechanisms. In those studies it is apparent that chemical interventions have unexpected or limited effects and the data obtained are difficult to interpret. Adrenalectomy is the only interventions that will allow one to precisely examine the role of HPA axis versus sympathetically mediated catecholamine release. Therefore, it is necessary to use total adrenalectomy for understanding the mechanism by which pollutant might induce metabolic disturbance.

SECTION C - Animal requirements

Describe the following animal requirements :

1. Indicate the number of animals required over the study period for this protocol. Please enter numbers only.

a. Animals to be purchased from a Vendor for this study: 192

b. Animals to be transferred from another LAPR:

LAPR Number that is the source of this transfer:

c. Animals to be transferred from another source:

d. Offspring produced onsite (used for data collection and/or weaned):

e. TOTAL NUMBER of animals for duration of the LAPR 192

2. Species (limited to one per LAPR): Rat(s)

3. Strain: WKY rat(s)

Describe special requirements for animals with altered physiological responses (e.g., genetically altered, aged)

None.

4. Sources of animals:

Charles River Laboratories, Inc.

5. Provide room numbers where various procedures will be performed on animals:

1. Rats will be housed in one of the animal housing rooms upon arrival Exemption 6 or other available room) on 5th floor and during non-exposure periods.

2. Prior to surgery, rats will be transferred in an original rack with rats housed in home cages to the third flood procedure room Exemption 6 Exemption 6. Once the animals are awake and in their home cages, rats will be moved back to the animal holding room.

3. During exposure, rats will be transferred in an original rack with rats housed in home cages to green floor inhalation exposure rooms (whole body exposures – Exemption 6 Exemption 6 Exemption 6. Once the exposures are complete rats will be transferred to their home cages in the same rack and moved to Exemption 6 Exemption 6 Exemption 6 for whole body plethysmography, temperature measurement and GTT/blood collection.

4. The day of necropsy, animals will be transferred to Exemption 6 Exemption 6 for necropsy using transfer cages with beta chips bedding and filtered cage tops.

6. Will any animals be housed in areas other than the animal facility longer than 12 hours? If so, state location. Such areas require prior IACUC approval as a satellite facility before LAPR can be reviewed.

No. *Room Numbers:* n/a

7. Describe any transportation and containment methods involved in moving animals between EPA buildings, or between EPA and other institutions (excluding any commercial shipments)

n/a

8. Describe any unusual housing or husbandry requirements, or acclimation requirements. Justify any treatment beginning less than 3 days after arrival.

None.

9. Describe special assistance requested of the animal contract staff, including procedures and dosing. NOTE, this request must be submitted separately to the Animal Resources Program Office (ARPO)

The days of surgical procedures, we will request 2-3 animal care personnel in assisting during animal recovery from anesthesia. There will be a requirement for bottled water, which will include salt water for adrenalectomized animals. The animal care personnel will be clearly instructed if additional assistance is needed during the study.

10. Housing and Enrichment.

The IACUC encourages the use of environmental enrichment whenever possible (see IACUC website for details). Provide details on how the animals will be housed, including type of cage (e.g., solid bottom or wire screen), bedding material, number of animals per cage, and environmental enrichment. Note that housing rodents individually without environmental enrichment requires justification.

All animals will double housed in solid bottom caging with beta chips bedding in A building during non-exposure periods as per the IACUC guidelines. Environmental enrichment including crinkled paper (EnviroDry) will be provided to allow the rats to nest.

SECTION D - Agents Administered to Animals

1. Identify all hazardous and non-hazardous agents to be administered to living animals. For agents requiring a Health and Safety Research Protocol (HSRP), provide the title of the approved HSRP for each such agent. If no protocol is required for an agent deemed potentially hazardous (e.g. nanoparticles, recombinant DNA), describe the safety precautions to be used.
Provide maximum dosing levels and route-appropriate LD50s (where available) for each agent used for dosing.

1) Ozone inhalation exposures: Ozone exposure will occur in whole body exposure chambers to a maximum of 0.8 ppm concentration.

The LC50 for ozone is 4.8 ppm in rats (4800 ppb/ 4 hours/ inhalation/ rat). HSP copy attached (Title: Small Animal Inhalation Exposures to Nitrogen Dioxide and ozone).

2) Saline: Pharmaceutical grade sterile saline will be injected subcutaneously (1 mL/kg) in vehicle control rats one day prior to the start of exposure, the first day prior to exposure and second day prior to exposure.

3) Clenbuterol: This beta adrenergic receptor agonist, will mimic the effect of circulating epinephrine, especially in the lung. Clenbuterol will be dissolved in saline at 1.0 mg/mL and administered

subcutaneously at 1mL/kg dose, one day prior to the start of exposure, the first day prior to exposure and second day prior to exposure. Oral LD50 value for Clenbuterol in rat is 159 mg/kg and intraperitoneal LD50 for rat is 67 mg/kg.

4) 1% carboxymethylcellulose in saline: 1% carboxymethylcellulose in pharmaceutical grade sterile saline will be injected intraperitoneally (1 mL/kg) as vehicle control for dexamethasone in rats, one day prior to the start of exposure, the first day prior to exposure and second day prior to exposure.

5) Dexamethasone, 5 mg/kg in 1 mL 1% carboxymethylcellulose in saline will be injected intraperitoneally (1 mL/kg) rats, one day prior to the start of exposure, the first day prior to exposure and second day prior to exposure. Acute oral toxicity (LD50) for rat is 7500 mg/kg and intraperitoneal LD50 is 2180 mg/kg.

6) Glucose: During GTT, pharmaceutical grade glucose will be injected intraperitoneally (maximum of 2 grams/kg body weight/10mL pharmaceutical grade saline). The 10ml/kg body weight volume has been used in published studies for rats. We will purchase pharmaceutical grade sterile glucose solution (40% concentration) from Sigma Aldrich and dilute to 20% using pharmaceutical grade saline.

7) Buprenorphine: This is a veterinary grade analgesic widely used in experimental studies during surgery. Rats will receive a total of three or more subcutaneous injections of Buprenorphine after the surgery at a recommended dose level of 0.02 mg/kg/mL in saline.

8) Meloxicam, an analgesic will be subcutaneously injected once in rats after they are awake from anesthesia. The recommended dose will be 0.2 mg/kg/mL in saline. This is a veterinary grade analgesic widely used in experimental studies during surgery.

Researchers will handle all agents in accordance with good industrial hygiene and safety practices. Lab coat, safety glasses and gloves will be worn when handling these chemicals. Drug preparations will be done in chemical safety hood. All animals will be monitored periodically during exposure.

2. Describe compounds to be administered to animals.

a. Are all substances pharmaceutical grade? If not, provide a scientific justification for the use of non pharmaceutical grade compounds.

Of 8 substances included in D1, Clenbuterol, dexamethasone and carboxymethyl cellulose are non-pharmaceutical grade. All published studies which we are following (some are attached have used the ultra pure research grade chemicals prepared in appropriate vehicles indicated in these studies. We will use previously published protocols for these drugs in the proposed studies. The use of these research grade drugs is necessary for comparing research results from our studies to those that have been published using these drugs.

b. Describe any plans to administer human or animal tissues, blood or body fluids to the animals in the LAPR. Provide information to assure that such material is pathogen free. Indicate what safety precautions are necessary for handling the material.

None.

c. Provide a statement regarding any safety precautions necessary for handling any of these materials.

Researchers will handle all agents in accordance with good industrial hygiene and safety practices. Lab coat, safety glasses and gloves will be worn when handling these chemicals. Drug preparations will be done in chemical safety hood. All animals will be monitored periodically during treatment/exposure.

NOTE: Any unresolved health/safety questions which arise during IACUC review of this LAPR will require consultation with the Safety, Health, and Environmental Management Office.

SECTION E - Personnel Training and Experience

1. Identify all project personnel conducting animal experimentation. Specify the techniques for

which they have responsibility, and their relevant training and experience. Additional personnel may be added to the table below as a group (by Division) for Category C procedures. By so doing you are giving assurance that these personnel have received all required training and are qualified to perform the Category C techniques requested.

Use this area to type in additional personnel information not available in the table drop-down lists:

Hint: *The names in the first 2 lines of the table below are filled automatically from the Principal Investigator & Alternate Contact fields. A new line will be made available when a name is selected & upon leaving the name field (i.e. tabbing or clicking in another field).*

NAME	ROLE	SPECIFIC RESPONSIBILITY	RELEVANT TRAINING
Exemption 6	Principal Investigator	Plan study, prepare protocols, and oversee the experiment. Assist in animal handling, testing, and necropsy.	Twenty years of experience working with rats at EPA and other institutions; all relevant NHEERL required training completed.
Exemption 6 Exemption 6 Exemption 6 Exemption 6	Technical Staff	Assist in animal handling, testing, and necropsy	Twenty years of experience working with rats at EPA and other institutions; all relevant NHEERL required training completed.
Exemption 6	Student	Plan study, prepare protocols, and oversee the experiment. Assist in animal handling, testing, and necropsy.	All relevant NHEERL required training completed. He will be mentored and supervised by the principal investigator during animal handling.
Exemption 6	Post-Doc	Plan study, prepare protocols, and oversee the experiment. Assist in animal handling, testing, and necropsy.	Four years of experience working with rats at EPA and other institutions; all relevant NHEERL required training completed.
Exemption 6	Post-Doc	Assist in animal handling, testing, and necropsy	Eight years of experience working with rats at various institutions including pregnant Long-Evans rats; all relevant NHEERL required training completed.
Exemption 6	Technical Staff	Assist in animal handling during in-life testing, exposure and plethysmography	Twenty years of experience working with rats at EPA and other institutions; all relevant NHEERL required training completed.
Exemption 6	Associate Principal Investigator	Assist in animal handling, testing, and necropsy	Twenty years of experience working with rats at EPA and other institutions; all relevant NHEERL required training completed.
Exemption 6	Post-Doc	Assist in animal handling, testing, and necropsy	Four years of experience working with rats at EPA and other institutions; all relevant NHEERL required training completed.
Exemption 6 Exemption 6 Exemption 6	Associate Principal Investigator	Assist in animal handling, testing, and necropsy	Twenty years of experience working with rats at EPA and other institutions; all relevant NHEERL required training

			completed.
Exemption 6	Technical Staff	Assist in animal handling, testing, and necropsy	Twenty years of experience working with rats at EPA and other institutions; all relevant NHEERL required training completed.
RTP-NHEERL	Tech Support	Category C Procedures	All NHEERL required training is complete.

SECTION F - Animal Breeding Colonies

This section pertains to the breeding of animals for maintenance of ongoing animal colonies. Do not include breeding that is part of experimentation and accountable under Section C.

Describe:

1. Estimated number of breeding pairs and liveborn per year None

2. Breeding protocols and recordkeeping n/a

3. Methods for monitoring genetic stability n/a

4. Disposition of all offspring and retired breeders that are not used in accordance with the procedures described in this LAPR n/a

SECTION G - Euthanasia

1. When will the animals be euthanized relative to experimental procedures?

Animals will necropsied for blood sample and tissue collections following terminal euthanasia, after 1 or 2 days or air or ozone exposure and within 10 days of surgical intervention.

2. Describe the euthanasia techniques:

Method(s): Euthanasia plus exsanguination

Agent(s): Pentobarbital injectable preparation, diluted with sterile saline to achieve maximum of 200 mg/mL concentration.

Dose (mg/kg): 200-250 mg pentobarbital/kg.

Volume: 1.0 – 3.0 mL/kg as needed

Route: Intraperitoneal

Source(s) of information used to select the above agents/methods:

Veterinary Staff
IACUC

3. Provide justification and references for any euthanasia agent or method that is not consistent with recommendations of the American Veterinary Medical Association (AVMA) Guidelines for Euthanasia (e.g., cervical dislocation or decapitation without anesthesia; cervical dislocation in rodents weighing more than 200 grams).

None

4. Describe how death is to be confirmed.

Vital organ section

SECTION H - Disposition of Used and Unused Animals

Describe the disposition of any animals remaining after project completion.

Transferred to another study

The IACUC encourages investigators to reduce the overall number of animals used at NHEERL. Would you consider transferring any unused animals from this LAPR to another approved LAPR?

● Yes ○ No

SECTION I - Assurances

1. Animals will not be used in any manner beyond that described in this application without first obtaining formal approval of the IACUC.
2. All individuals involved in this project have access to this application, are aware of all EPA policies on animal care and use, and are appropriately trained and qualified to perform the techniques described.
3. Thorough consideration of the three "R"'s (Replacement, Reduction, Refinement) has been given, as applicable, to a. the use of animals, and b. procedures causing pain or distress (with or without analgesia/anesthesia), including death as an endpoint. The minimum number of animals required to obtain valid experimental results will be used.
4. The Attending Veterinarian has been consulted in regard to any planned experimentation involving pain or distress to animals.
5. The IACUC and Attending Veterinarian will be promptly notified of any unexpected study results that impact the animals' well-being, including morbidity, mortality and any occurrences of clinical symptoms which may cause pain or indicate distress.
6. All procedures involving hazardous agents will be conducted in accordance with practices approved by the Safety, Health, and Environmental Management Office.
7. I certify that I am familiar with and will comply with all pertinent institutional, state and federal rules and policies.
8. The IACUC has oversight responsibilities for animal care and use, and may request consultation or feedback regarding the conduct of in vivo procedures, progress and accomplishments, and any problems encountered.

EPA Principal Investigator	Certification Signature Date
Exemption 6 Exemption 6	05/09/2016

Submitted: 05/09/2016

Certification:

Certification by EPA Supervisor (Branch Chief or Division Director) that the project described herein has been reviewed and approved on the basis of scientific merit:

Branch Chief/Division Director	*Approval Date*	*Phone Number*	*Division*	*Mail Drop*
Exemption 6	05/09/2016 by Exemption 6 Exemption 6 /RTP/USEPA/US	Exemption 6 *Lotus Notes Address* Exemption 6 Exemption 6 /RTP/USEPA/US	*Branch*	MD *Submitted to Branch Chief for Approval* 05/09/2016 02:06 PM

ATTACHMENTS

19-05-002 PI resp.pdf
19-05-002 discussion.pdf
Experimental design for LAPR r1.pptx
2016 ADREX study protocol.xlsx
clenbuterol.pdf
Dex-MSDS.pdf

Actions

First Update notification sent: 04/03/2017
Second Update notification sent: 05/02/2017
First 2nd Annual notification sent:

Second 2nd Annual notification sent: 04/03/2018
1st Expiration notification sent:
2nd Expiration notification sent:

History Log: